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Abstract

The central nervous system (CNS) and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. Organophosphates, such as the nerve gas sarin, are powerful irreversible inhibitors of ChE, leading to neurotoxicity, seizures, and death. Because of the ease and low cost of production, sarin gas is a tool of mass destruction in the hands of terrorist groups and rogue nations. While people in the immediate vicinity of sarin attack may receive neurotoxic doses, people away from this area are likely to receive subclinical exposures. Even subclinical doses of sarin cause subtle changes in the brain, and subclinical exposure to sarin have been proposed as an etiology to the Gulf War Syndrome. Our preliminary experiments suggest that low doses of sarin are highly immunosuppressive, and suppress glucocorticoid production. The effects of sarin exposure on the immune system are attenuated by ganglionic blockers and decreased glucocorticoid level may be a biomarker for cholinergic toxicity. Future experiments are designed to understand the mechanism of sarin-induced immunotoxicity. The study may identify novel biomarkers of nerve gas exposure, and suggest therapeutics to treat the immunotoxicity.

INTRODUCTION: The central nervous system (CNS) and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. Organophosphates, such as the nerve gas sarin, are powerful irreversible inhibitors of cholinesterases, and might cause neurotoxicity, seizures, and death. Because of the ease and low cost of production, sarin gas is a tool of mass destruction in the hands of terrorist groups and rogue nations. Even a subclinical dose of sarin causes subtle changes in the brain. Increasing evidence suggests that the major health effects of sarin are primarily through its effects on the CNS. Although unproven, subclinical exposure of Gulf War veterans to sarin has been implicated in the development of the Gulf War syndrome (GWS). Interestingly, the symptoms of GWS are similar to diseases that result from impaired immune/inflammatory responses and include muscle fatigue, general malaise, myalgia, impaired cognition, ataxia, headaches, fever, joint pain, skin rash, gastrointestinal and sleep disturbances, and respiratory difficulties. Previous studies from our lab showed that repeated exposure to low doses of sarin (0.2-0.4 mg/m^3) for 5 days suppressed T cell proliferation to mitogens and antigens, and inhibited the T cell antigen receptor (TCR)-induced rise in intracellular Ca^{2+} concentration. In addition, sarin dramatically decreased serum corticosterone (CORT) levels. Moreover, effects of sarin on T cell proliferation were blocked by the ganglionic blocker, chlorisondamine. These results suggested that the effects of sarin on T cell responsiveness are mediated through the autonomic nervous system (ANS). In order to understand the mechanism of cholinergic immunotoxicity in general, and of sarin in particular, the following tasks were proposed in the grant application:

Task 1. To determine whether cholinergic agents require access to the CNS to alter the immune response and corticosterone levels (Months 1-18).

a. Ascertain whether cholinergic agents (pyridostigmine bromide, physostigmine, sarin, edrophonium) cause immunosuppression, and whether the development of immunosuppression requires access to the CNS (1-4 months).
b. Determine whether cholinergic agents depress corticosterone levels; if so, if it is blocked by ganglionic blockers (5-11 months).
c. Establish the kinetics of corticosterone suppression by sarin (12-18 months).

Task 2. Determine the steps in the antigen-induced T cell signaling pathway that are affected by low-dose sarin inhalation (19-29 months).

a. Determine the effects of sarin on Src-like preteen tyrosine kinases (Fyn and Lck) (19-22 months).
b. Examine the effects of sarin on PLC-γ1 (23-26 months)
c. Investigate the effects of sarin on intracellular calcium stores (27-29 months).

Task 3. To ascertain the role of the sympathetic autonomic nervous system in sarin-induced immunotoxicity (30-39 months).

a. Examine whether sarin activates the HPA axis (30-31 months).
b. Examine whether chemical or surgical sympathectomy abrogates the immunological effects of sarin (32-36 months).
c. Investigate whether β-adrenoceptor antagonists block the effects of sarin on T cell proliferation (37-38 months).

Task 4. What roles do muscarinic and nicotinic acetylcholine receptors play in sarin-induced immunotoxicity? (39-48 months).

a. Determine the effects of sarin on changes in the acetylcholine esterase activity in various brain regions (39-40 months).
b. Establish whether sarin affects the density of nicotinic acetylcholine receptors in various brain regions (41-43 months).
c. Determine whether sarin affects the immune system in muscarinic or nicotinic acetylcholine receptor knockout mice (44-48 months).

BODY:

1. **Summary of the Last Progress Report:** We previously reported Kalra et al. (2002) that sarin-treated rats have significantly lower serum levels of corticosterone and decreased T-cell immunity, and these effects are mediated through the CNS. The mechanism of sarin-induced immunotoxicity is largely unknown. Toward understanding the mechanism of sarin induced immunotoxicity, F344 rats were exposed to subclinical doses of sarin (0.4 mg/m^3) for 1h/day for 5 days. The following is a summary of the results that were submitted in the last years Progress Report:

(i) Cholinergic compounds that cross the blood-brain-barrier (BBB) inhibit the immune response when given by inhalation or intracerebroventricularly (ICV), however, poorly BBB permeable cholinergic compounds, unless given in very high doses, are immunosuppressive only when administered directly into the brain (i.e., via ICV).

(ii) Cholinergic compounds that cross the BBB also decrease serum corticosterone (CORT) levels, and as with the immune response the inhibitory CORT effects of sarin are attenuated by pretreatment with the ganglionic blocker - chlorisondamine. Similarly, sarin-induced inhibition of antigen- and mitogen-driven T cell proliferation was moderated by pretreatment with chlorisondamine. These results were published in Langley et al. (2004), and suggest that sarin affects the immune and adrenal responses through the autonomic nervous system.

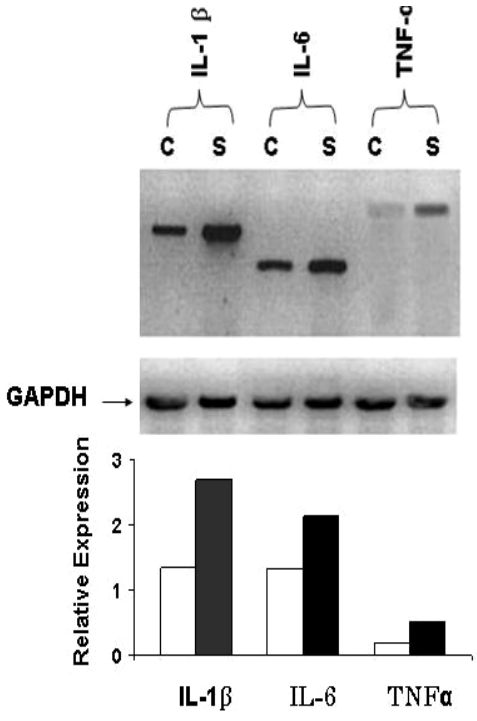

Fig. 1. *A single subclinical inhalaion of sarin increases the mRNA levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α. A representative RT-PCR blot with mRNA from control (C) and sarin-treated (S) rats at 24 h after sarin treatment (top panel)*
The differences in the expression were quantitated by densitometry of the gel (bottom panel).

(iii) Kinetic studies on the effects of sarin on immune parameters suggested that most immunosuppressive and proinflammatory (increased proinflammatory cytokine expression) effects of low-dose sarin persist for 1-2 wk post sarin exposure.

2. Recent Experimental Results

The following describe our recent results with rats exposed repeatedly to a subclinical dose (0.4 mg/m^3 for 1 h/d for 5 d) or to a single high dose (2.0 mg/m^{3}for 1h) of sarin. The latter was added to simulate a realistic battlefield situation or chemical terrorism attack.

(a) Experiments related to the effects of sarin on T cell function

We have demonstrated that subclinical doses of sarin suppress the antibody response (Kalra et al., 2002), but it also increases the level of proinflammatory cytokines IL-1β, TNF-α, and IL-6 in the brain (Henderson et al., 2000). Rats exposed to 1 Ltc_{50} sarin also develop frank lung inflammation (Pant et al., 1993); therefore, it was possible that sarin has both immunosuppression (inhibition of adaptive immunity) and proinflammatory effects (stimulation of innate immunity). We recently observed that stimulation of T cells through the TCR activates two major signaling pathways: Src-like protein tyrosine kinases (PTK)-dependent increase leading to increase in $[Ca^{2+}]_i$ and Src-independent activation of the transcription factor NFκB (Razani-Boroujerdi et al., in preparation). NFκB is the most potent transcription factor for the transcription of the

proinflammatory cytokine genes IL-1β, TNF-α, and IL-6. Sarin inhibits the T cell antigen receptor (TCR)-mediated increase in the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Kalra et al., 2002)., and the expression of these cytokines is increased in the brains of sarin treated animals (Henderson et al., 2002). To delineate the mechanism by which sarin modulates both adaptive and inflammatory responses, we examined its effects on T cell function and lung inflammation. The results of these experiments are summarized below:

(i) **Sarin induces the expression of proinflammatory cytokines in the lung**

Because acute exposure to high doses of sarin (1 Lct_{50}) caused serious lung inflammation in rats (Pant et al., 1993), we examined the expression of the proinflammatory cytokines IL-1β, TNF-α and IL-6 in the rat lung after single inhalation of a subclinical dose of sarin (0.4 mg/m^3 for 1 h). Fig. 1 shows a representative RT-PCR blot suggesting that sarin increases the transcription of proinflammatory cytokines in the lung.

(ii) **Sarin increases nuclear translocation of NFκB**

NFκB is the most potent transcription factor for the transcription of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6). To determine whether sarin affects the transcription of proinflammatory cytokines by upregulating the levels of NFκB, nuclear extracts from bronchoalveolar lavage (BAL) cells were obtained at 24 h after a single exposure to sarin (1 h at 0.4 mg/m^3). Fig. 2A shows that sarin strongly increases the nuclear content of NFκB that is super-shifted by an anti-p50 subunit-specific antibody. Moreover, generally the activation of NFκB requires the activation of the MAP kinase pathway, and phosphorylation of ERK is a major event in the activation pathway. T cells were obtained from control and sarin-treated rats and stimulated with anti-T cell receptor (TCR) + anti-CD28 antibodies. Cell extracts were assayed for ERK and phospho-ERK (pERK) by Western blot analysis using ERK- and pERK-specific antibodies. Results presented in Fig. 2B show that although the total amount of ERK is not affected by a single subclinical dose of sarin, ERK is activated (i.e., increased pERK content) by sarin treatment. The stimulatory effects of sarin on ERK activity is partially blocked by pretreatment with the ganglionic blocker chlorisondamine (CHL).

(iii) **Sarin increases the expression of Substance P (SP).**

Sarin affects a number of inflammatory parameters in the lung. SP is known to promote lung inflammation through production of proinflammatory cytokines via NFκB activation (Okaya et al., 2004; Azzolina et al., 2003). Moreover, organophosphate pesticides induce SP. Therefore, we determined whether sarin affects SP transcription in the lung. Indeed, a single subclinical exposure to sarin significantly increased the transcription of SP at 24 h after sarin exposure (Fig. 3). Moreover, preliminary experiments suggest that immunoreactive SP levels in BALF from sarin-treated rats are increased by approximately 3-fold (data not shown; these results need to be confirmed). Thus, sarin stimulates SP expression in the lung, and the increased production of SP might contribute to sarin-induced NFκB activation and lung inflammation.

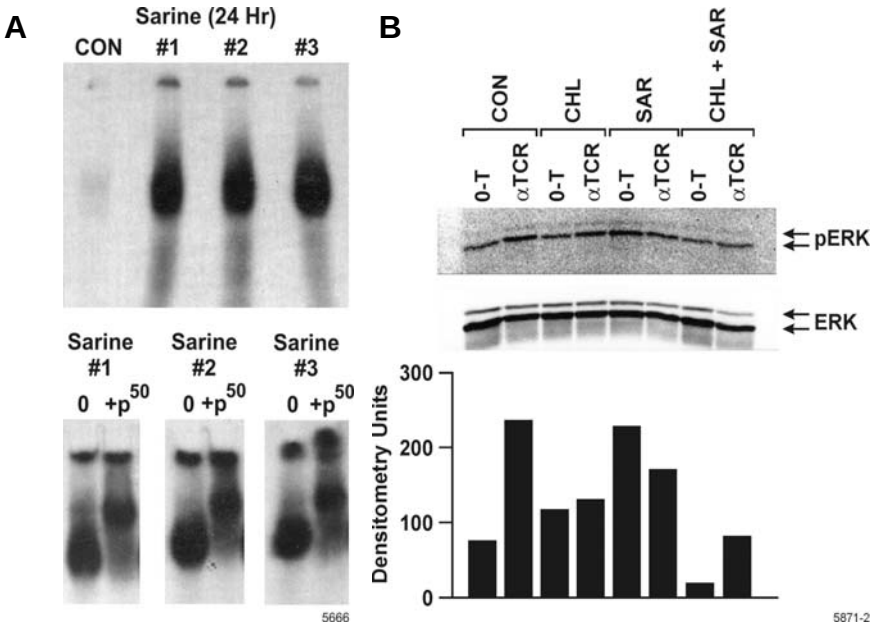

Fig. 2. *Sarin activates NFκB and ERK. **A**: Nuclear extracts from BAL cells from a control (CON) rat and three sarin-treated rats (samples #1, 2, 3) were analyzed by the electrophoretic mobility shift assay (top panel), and sarin-treated samples were super-shifted with p50 subunit-specific antibody (bottom panel). **B**: Splenic T cells were isolated from control (CON) and sarin-treated rats (SAR). Some animals from CON and SAR groups were also given the ganglionic blocker chlorisondamine (CHL; Kalra et al., 2002) 7 days prior to sarin inhalation. T cells were cultured with isotype control (0T) or with anti-αβ TCR + anti-CD28 (α-TCR) antibodies for 1 h. Cell extracts were run on gel electrophoresis, blotted, and probed with ERK-specific antibodies (middle panel), with pERK after stripping the blots (top panel). Bottom panel shows the densitometric analysis of pERK from the top panel figure.*

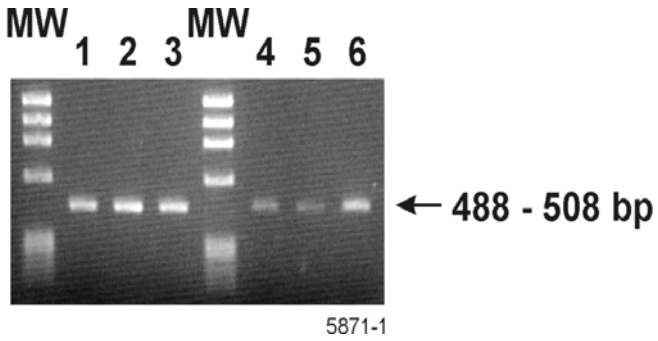

Fig. 3. *Sarin stimulates SP transcription. Rats were exposed to a single subclinical dose of sarin. mRNA was isolated from three sarin-treated (samples 1, 2, 3) and three control (samples 4, 5, 6) rats for RT-PCR analysis. The results of RT-PCR showing increased levels of SP-specific mRNA in sarin-treated lungs are presented in the figure. MW: Molecular weight markers.*

(iv) Sarin increases airway resistance response to methacholine

Bronchoconstriction is one of the serious consequences of exposure to high-dose sarin. High-dose exposure to organophosphate pesticides not only induces bronchoconstriction but increases the density of muscarinic receptors in the lung, making them more sensitive to muscarinic agonist. To ascertain whether exposure to sarin increases the airway resistance response to muscarinic agonists, rats were exposed to a single dose of 0.5 LC_t50 sarin. The airway resistance of these animals was determined by plethysmography immediately following sarin exposure. Fig. 4 shows that sarin significantly increases the response of lungs to methacholine-induced bronchoconstriction. Experiments are currently underway to determine whether the increased sensitivity to methacholine is associated with changes in the density of muscarinic receptors.

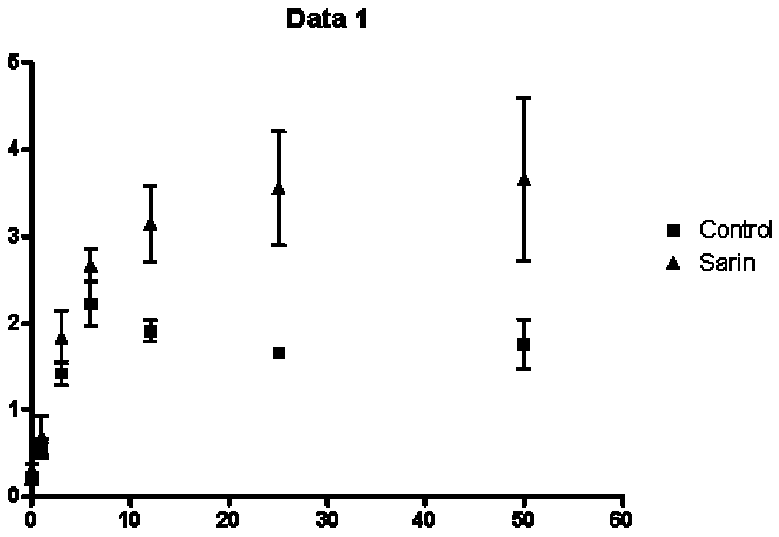

Fig. 4. ***Sarin increases response to methacholine.*** *F344 rats were exposed through inhalation to sarin (0.5 LC_t50) for 1 h, and subjected to plethysmography as described in General Methods. It is clear that the airway resistance is significantly higher in sarin-treated animals (X-axis, methacholine concentration; Y-axis, airway resistance). The readings are an average of six animals in each group.*

KEY RESEARCH ACCOMPLISHMENTS SINCE THE GRANT INITIATION

- Exposure to subclinical doses of sarin suppresses the immune system and glucocorticoid production, and the effects are at least partially ameliorated by pretreatment with ganglionic blockers. Preliminary experiments (to be confirmed) suggest that changes is the corticosterone levels might reflect changes in the plasma ACTH levels.
- Cholinergic agents that cross the blood-brain-barrier cause immunotoxicity similar to sarin.
- Most effects of sarin are temporary (i.e., lasting for 1-2 wk).
- Sarin causes airway resistance, and upregulates the markers of neurogenic inflammation in the lung.
- Sarin increases MAP kinase (e.g. ERK) and NFκB activities in T cells; changes in the ERK and NFκB could explain the increase in the inflammatory response and production of proinflammatory cytokines.
- Ganglionic blockers may have therapeutic value in the treatment of cholinergic immunotoxicity, and decreased serum glucocorticoid level is a potential biomarker for exposure to sarin and other BBB-crossing cholinergic agents.

REPORTABLE OUTCOMES

The above described new results have not yet been published. We are currently putting together two manuscripts to cover these data:

1. Pena-Philippides, J.C., N.M. Mishra, R.J. Langley, S. Razani-Boroujerdi, R. Kalra, S.P. Singh, and M.L. Sopori. Kinetics of sarin immunotoxicity: Immunotoxicity of low-dose sarin is transitory and changes in the plasma corticosterone follow those for ACTH. (In preparation).

2. Pena-Philippides, J.C., S. Razani-Boroujerdi, S.P. Singh; R.J. Langley, N.M. Mishra, J.S. Nandi, and M.L. Sopori. Sarin causes bronchoconstriction and neurogenic inflammation in the lung and upregulates the expression of M1, M3, and M5, but not of M2 and M4 muscarinic receptors. (In preparation).

CONCLUSIONS

Subclinical exposure to cholinergic agents, such as sarin, pesticides, and other organophosphates suppress the immune system, and this immunotoxicity is dependent on their ability to cross the BBB. The effects are mediated through the autonomic nervous system and are at least partially overcome by ganglionic blockers. Cholinergic neurotoxicity also suppresses glucocorticoid production; this effect follows the changes in the plasma ACTH levels. Changes in these markers may be biomarkers for cholinergic toxicity. The Increased brain levels of proinflammatory cytokines might cause some early symptoms of the Gulf War syndrome, and their increased expression in the lung may result from neurogenic inflammation. Ganglionic blockers may have some therapeutic value in ameliorating the immunosuppressive and inflammatory effects of sarin.

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